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Analysis of polymyxin B sulfate by capillary zone electrophoresis with cyclodextrin as additive
Method development and validation

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Abstract

A capillary zone electrophoresis method for analysis of polymyxin B sulfate is described. In this method, triethanolamine (TEA)–phosphate buffer at pH 2.5 was employed to reduce the adsorption of analyte onto the capillary wall. Methyl-β-cyclodextrin (M-β-CD) and 2-propanol (IPA) were found to be necessary for selectivity enhancement. In order to optimize the method and to control its robustness, a central composite design was performed with four parameters, i.e. concentration of M-β-CD, TEA, IPA and buffer pH. The optimal separation conditions were as follows: capillary, 55 cm (50 μm I.D., 47 cm effective length); 130 m*M* TEA–phosphate buffer (pH 2.5) containing 5 m*M* M-β-CD and 5% IPA; 24 kV (51 μA) applied voltage; column temperature, 20°C. Further, linearity and limits of detection quantification were examined. Three commercial samples were analyzed quantitatively. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Validation; Pharmaceutical analysis; Polymyxin B sulfate; Antibiotics; Cyclodextrins

1. Introduction

Polymyxin B belongs to the group of polymyxin antibiotics isolated from various strains of *Bacillus polymyxa* and related species. Its sulfate salt is used for the treatment of infections caused by gram-negative bacteria. Polymyxin B is a mixture of several closely related compounds differing from each other in structure mainly by a fatty acyl residue [1]. The known components of polymyxin B are polymyxin B_1, B_2, B_3 and B_4 [1]. They have a general structure composed of a cyclic heptapeptide moiety and a tripeptide side chain, N-acylated by a fatty acyl residue (Fig. 1).

Several methods have been used in the analysis of polymyxin. Counter current distribution was used for preparative separation of polymyxin [2]. Thin-layer chromatography (TLC) [3] and liquid chromatography (LC) [4–12] were used to demonstrate the complex composition of this substance. Gas chromatography (GC) was used for analysis of the fatty acids after hydrolysis [13]. Microbiological assay was employed for testing the potency of polymyxin B_1 and B_2 [14]. Recently, capillary electrophoresis (CE) was also used for polymyxin separation [15]. In this method, a zwitterionic surfactant, 3-(*N*,*N*-di-

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R→L-DAB→L-Thr→L-DAB→L-DAB→L-DAB→D-Phe→L-Leu→L-DAB→L-DAB→L-Thr (cyclic to L-DAB); each L-DAB with γ-NH$_2$

Polymyxin B_1 R = (+)-6-methyloctanoyl

B_2 R = 6-methylheptanoyl

B_3 R = octanoyl

B_4 R = heptanoyl

Fig. 1. Chemical structure of known components of polymyxin B. DAB, α,γ-diaminobutyric acid; Thr, threonine; Phe, phenylalanine; Leu, leucine.

methylhexadecylammonium) propanesulphonate (PAPS), was added to the running buffer as pseudo-stationary phase. Selectivity was acceptable, but the separation showed poor efficiency and resolution due to peak tailing. These may cause errors in quantitative analysis.

Cyclodextrins (CDs) are widely used as chiral selectors in enantiomer separation by CE [16–18]. In addition, CDs have also been used as a buffer additive to enhance the achiral selectivity in CE, e.g. of peptides [19], pharmaceuticals [20,21], plant growth regulators [22], aromatic positional isomers [23] and environmental samples [24–27]. This subject was recently reviewed by Luong and Nguyen [28].

The aim of this work was to develop a new CE method that can be applied for quantitative analysis and thus quality control. Method validation, including robustness, was performed and four commercial samples were analyzed quantitatively.

2. Experimental

2.1. Apparatus

CE was performed on Spectraphoresis 1000 equipment (Thermo Separation Products, Fremont, CA, USA) which was controlled by CE software (Version 3.0.1) operated under IBM OS/2 (Version 1.2). The untreated fused-silica capillary (375 μm O.D.×50 μm I.D.) of 55 cm length (47 cm to detection window) was from Polymicro Technologies (Phoenix, AZ, USA). Buffer pH was measured with a Consort C-831 pH meter (Turnhout, Belgium).

2.2. Chemicals and reagents

Triethanolamine (TEA) (analytical grade) was from Merck (Darmstadt, Germany). 2-Propanol (IPA) (HPLC grade) and phosphoric acid (analytical grade) were from Riedel-de Haën (Seelze, Germany). Methanol (HPLC grade) and acetonitrile (HPLC grade) were from Rathburn (Walkerburn, UK) and Biosolve (Valkenswaard, Netherlands), respectively. α-CD and β-CD were from Acros (Geel, Belgium). Methyl-β-cyclodextrin (M-β-CD) with an average degree of substitution of 1.8 was purchased from Aldrich (Bornem, Belgium). Polymyxin B sulfate was from Ludeco (Brussels, Belgium). The sulfate salts of purified samples of polymyxins B_1, B_2, B_3 and B_4 were prepared by semi-preparative LC, and the molecular masses were determined by mass spectrometry [29].

2.3. Procedure

Before use, a new capillary was treated with 0.1 M NaOH for 2 h. The capillary was conditioned at the beginning of each day with 0.1 M NaOH for 5 min,

followed by 0.5 *M* H_3PO_4 for 5 min, water for 10 min and then with running buffer for 5 min. Capillary temperature was controlled at 20°C. The samples were hydrodynamically injected for 4 or 8 s (0.75 p.s.i.; 1 p.s.i.=6894.76 Pa). Between two runs, the capillary was flushed with running buffer for 5 min. Buffers were prepared starting from 0.5 *M* TEA stock solution, the buffer pH was adjusted with 1.5 *M* phosphoric acid to the desired value, and IPA was added. The solution was then made up to the mark with water. A certain amount of cyclodextrins was dissolved in this buffer to give the run buffer for the inlet buffer vial, while the outlet buffer vial contained no cyclodextrin. All the solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through 0.2 μm nylon filters (Alltech, Laarne, Belgium) prior to use. Stock solution of polymyxin sulfate (3 mg/ml) was prepared by dissolving appropriate amounts of the material in water. The peaks were identified by spiking with the purified samples. Detection was performed at 195 nm, the wavelength at which polymyxin has a maximum absorbance in the CE buffer system.

2.4. Software

Experimental design and optimization were performed using Modde 4.0 software (Umetri, Umeå, Sweden).

3. Results and discussion

3.1. Method development

One of the undesirable problems encountered in CE separation is adsorption of the positively charged analyte onto the capillary wall, which can cause peak tailing and poor resolution. Since all the main components of polymyxin B possess five unmasked amino groups, the adsorption of polymyxin B onto the capillary wall is a potential problem. A simple way to reduce such adsorption is by using a buffer with low pH and relatively high concentration [30]. It was found that a phosphate buffer (pH 2.5) composed of TEA and phosphoric acid was better than the commonly used monobasic sodium phosphate buffer, because the former was more effective in reducing the adsorption of analyte onto the capillary wall, generated less Joule heat and matched better the mobility of the analytes than the latter. Another problem was that the polymyxin B components have equal or nearly equal mass-to-charge ratios. Consequently, their electrophoretic mobilities are quite similar, resulting in poor separation selectivity.

In preliminary work, the ionic surfactant cetyltrimethylammonium bromide (CTAB) (5 m*M*) as well as non-ionic surfactants Brij 35, Tween 80, Tween 20 and Triton X-100 (0.1 and 0.5% each) were tested individually. However, the improvement in selectivity was rather limited because most of the peaks still overlapped one another. The selectivity was improved dramatically when 5 m*M* α-CD, β-CD and, especially, M-β-CD was added to the buffer. No other CDs were tested and only M-β-CD was selected for further investigation. The enhancement of the selectivity might be attributed to the ability of CD to selectively include the fatty acyl portion of the polymyxin B into its hydrophobic cavity. It was found that further improvement of the separation for the minor peaks around polymyxin B_1 was needed. However, no obvious improvement was achieved by varying the concentration of M-β-CD from 1 to 7 m*M* (keeping the buffer concentration at 100 m*M*), or by varying the concentration of the buffer from 50 to 150 m*M* (keeping the concentration of M-β-CD at 5 m*M*).

In CE, organic solvents are commonly used as buffer modifier to tune the selectivity by altering the polarity and the viscosity of the buffer. Therefore, three organic solvents, methanol, acetonitrile and IPA [5 and 10% (v/v) each], were tested as buffer additive for further improvement of the selectivity. It was found that IPA gave the best overall improvement in selectivity, because two minor peaks that co-migrated with polymyxin B_1 and another pair of minor peaks before polymyxin B_1 were now separated.

Furthermore, the effect of capillary length and applied voltage (range examined: 44–60 cm and 18–26 kV, respectively) on separation was also investigated. It was found that a capillary with an effective length of 47 cm combined with an applied voltage of 24 kV could give a satisfactory separation in a reasonable analysis time.

Table 1
Variables and their ranges studied

Variable	Low level (−1)	Central value (0)	High level (+1)
M-β-CD concentration (m*M*)	1	4	7
TEA concentration (m*M*)	100	125	150
Buffer pH	2.3	2.5	2.7
IPA concentration (%, v/v)	3	5	7

3.2. Optimization and robustness

In CE, when more than one buffer modifier is employed, it is difficult to obtain optimal conditions using the commonly used step-by-step optimization procedure, i.e. one parameter is varied while the others are held constant. This is because such a procedure only takes the independent effect of each parameter into account, while sometimes the interaction of two parameters may play an important role in separation selectivity. Therefore, in order to obtain the overall optimal conditions, the interaction between different parameters should be considered. An experimental design based on chemometrics has been established as useful for resolving such a problem [31]. Robustness is an important feature of an analytical method, which has to be verified. Experimental design can also be used for this purpose [32–34]. The basic idea of experimental design is to vary all relevant parameters simultaneously over a set of planned experiments and then connect the results by means of a mathematical model. This model is then used for interpretation, prediction and optimization as well as robustness evaluation.

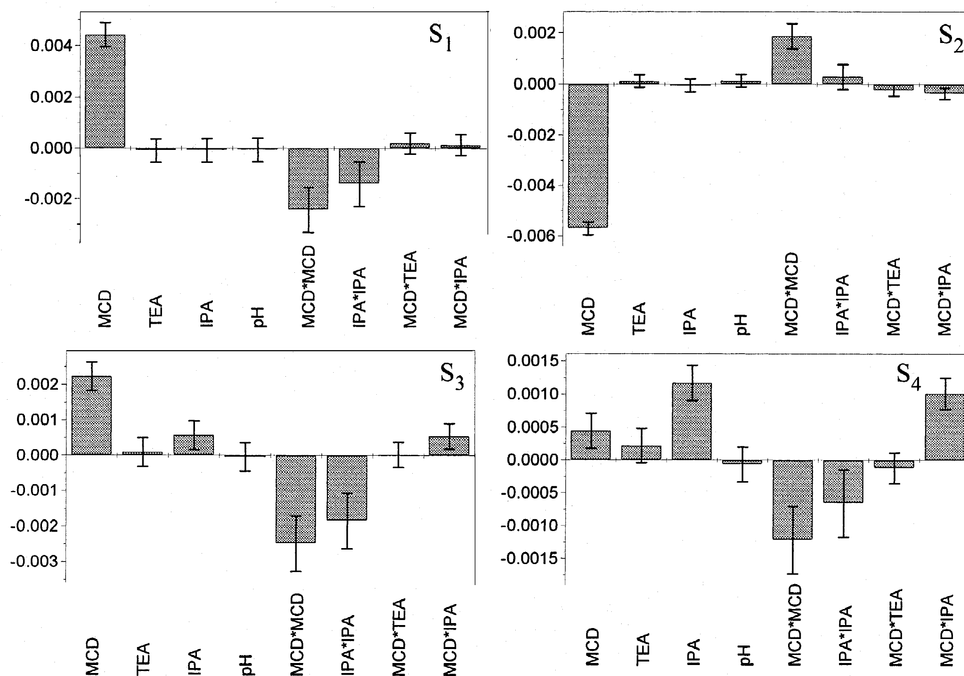

Fig. 2. Regression coefficient plot for the separation selectivity. S_1, selectivity between B_2 and a minor peak eluted before it; S_2, selectivity between B_2 and a minor peak eluted after it; S_3, selectivity between B_1 and a minor peak eluted before it; S_4, selectivity between B_1 and a minor peak eluted after it.

In the present case, four variables and four responses were involved in the experimental design. Variables and their ranges studied are summarized in Table 1. The high and low values of each variable were defined based on preliminary experiments. Since polymyxin B_1 and B_2 are the major components, four responses, S_1, S_2, S_3 and S_4, corresponding to the separation selectivity between main components (B_1 and B_2) and adjacent minor peaks were used as optimization criteria. The selectivity was calculated by the equation: $S = t_2/t_1$, where t_1 and t_2 are the migration times of the peak pairs.

A central composite design was used for the purpose of this study. This experimental design needed 27 experiments in total ($2^k + 2k + 3$, where k is the number of variables) including three center points. The center points are very important because they give information concerning the repeatability of the design. The collected experimental data were fitted by a partial least square (PLS) model with which several responses (three or more) can be dealt with simultaneously, to provide an overview of how all the factors affect all the responses. For four responses of the model, R^2 and Q^2 values were over 0.91 and 0.84, respectively, implying that the data fitted well with the model. Here, R^2 is the fraction of the variation of the response that can be modeled and Q^2 is the fraction of the variation of the response that can be predicted by the model. R^2 and Q^2 values close to 1 indicate an excellent model.

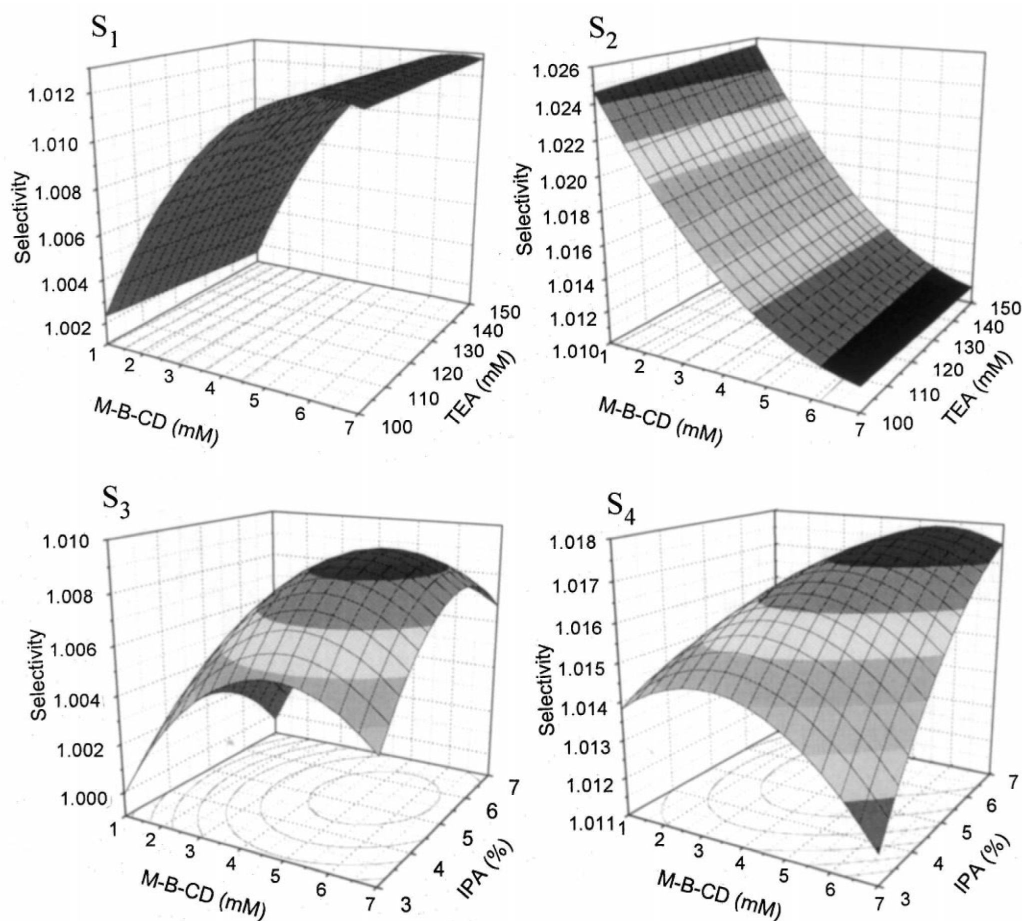

Fig. 3. Response surface plot showing the selectivity as a function of significant separation parameters. S_1, selectivity between B_2 and a minor peak eluted before it; S_2, selectivity between B_2 and a minor peak eluted after it; S_3, selectivity between B_1 and a minor peak eluted before it; S_4, selectivity between B_1 and a minor peak eluted after it.

The relationship between a response y and the variables x_i, $x_j \ldots$ can be described by a Taylor expansion:

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \beta_{ii} x_i^2 + \beta_{jj} x_j^2 + \cdots + E$$

where β is the regression coefficient and E is the overall experimental error. The square term of each variable describes the non-linear effect on the response, and the cross term of the two different variables describes the effect of their interaction on the response. Fig. 2 shows the regression coefficient plot for four responses. The 95% confidence interval was expressed in terms of the error bar over the coefficient. If the coefficient is smaller than the interval, the variation of the response caused by changing the variable is smaller than the experimental error. Therefore, the variable is considered not to be significant. During refinement of the model, some interactions, which had been established not to be significant, were removed. Thus in Fig. 2 it can be seen that, for all responses, the effect of the buffer pH on selectivity was not significant in the studied range. This may be due to the fact that pH 2.5 is too far below any pK_a value of polymyxin B components. M-β-CD, on the other hand, has a significant but non-linear effect on the separation selectivity (see variable*variable item in Fig. 2). Except for S_2, M-β-CD has a positive effect on selectivity. IPA shows a positive significant effect on S_3 and S_4. In addition, the interaction between IPA and M-β-CD has a significantly positive effect on S_4. TEA concentration only shows a significant effect on S_4.

The response surface plots constructed by plotting separation selectivity as a function of important variables are shown in Fig. 3. Since the optimal conditions predicted by the model for each peak pair were not completely the same, the overall one was obtained by balancing. Thus the overall optimal conditions are as follows: 130 mM TEA (pH 2.5) containing 5 mM M-β-CD and 5% IPA. A typical electropherogram for a commercial sample is shown in Fig. 4.

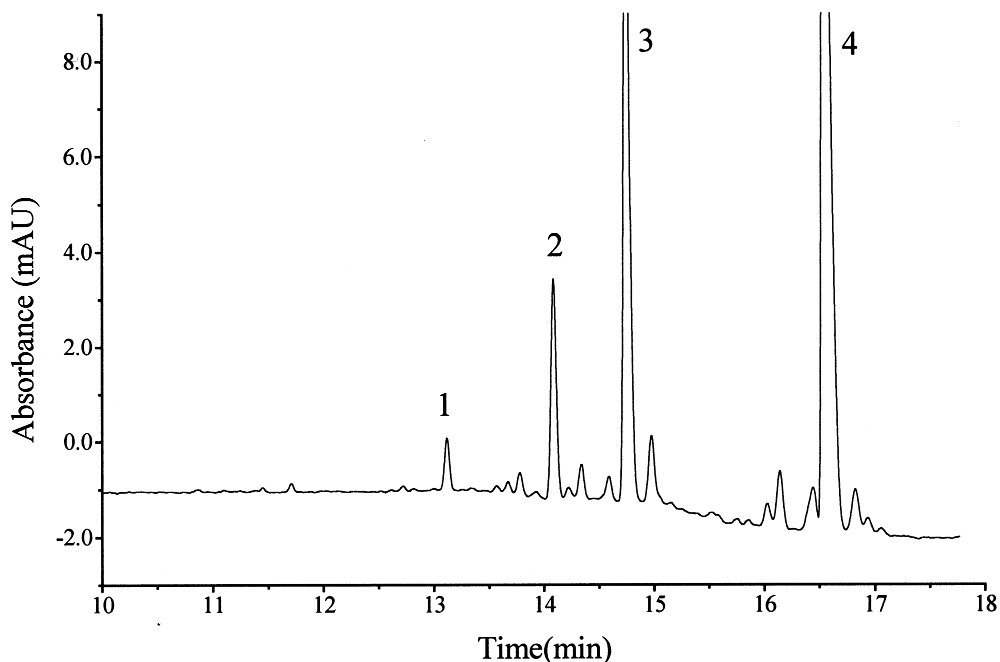

Fig. 4. Typical electropherogram of a commercial sample of polymyxin B sulfate. Conditions: background electrolyte, 130 mM TEA–phosphate buffer (pH 2.5) containing 5% IPA and 5 mM M-β-CD; Applied voltage, 24 kV; current, 51 μA. Peaks: 1 = polymyxin B_4, 2 = polymyxin B_3, 3 = polymyxin B_2, 4 = polymyxin B_1. The amount of polymyxin B_1 was determined as 3.0 ng.

Table 2
Quantitative features for polymyxin B_1 [a]

Parameter	
Intra-day repeatability (n=6)	
Migration time	RSD 1.2%
Corrected area	RSD 1.3%
Inter-day repeatability (n=6 days)	
Migration time	RSD 1.4%
Corrected area	RSD 2.4%
Linearity [b]	$y = 15\,850x + 388$, $r = 0.998$, $S_{y,x} = 626$
LOD ($S/N = 3$) [c]	0.0012 mg/ml (0.06%, related to 2 mg/ml)
LOQ ($S/N = 10$) [c], RSD=7.98% ($n = 7$)	0.0023 mg/ml (0.1%, related to 2 mg/ml)

[a] Injection volume was calculated as 11.2 nl.
[b] Injection for 4 s; y=corrected area, x=concentration in mg/ml, number of concentration points=6, each point was injected in triplicate.
[c] Injection for 8 s.

Compared with the result of LC [12], the separation selectivity is comparable, but the analysis time is about three times shorter than with the latter.

It can be seen from the response plots (Fig. 3) that when M-β-CD varies from 4 to 6 m*M*, IPA from 4.5 to 6% and TEA from 120 to 140 m*M*, the response surface is relatively flat. This implies that the separation method developed here is robust in the above-mentioned range.

3.3. Quantitative analysis

The quantitative features of this method were investigated with polymyxin B_1, because only polymyxin B_1 with a purity of 95% was available in sufficient amount. The linearity (peak area) of polymyxin B_1 was examined in the range from 0.013 to 0.95 mg/ml (six points), each point being injected in triplicate. Limit of quantitation (LOQ) and limit of detection (LOD) corresponding to signal-to-noise ratios of 10 and 3, respectively, were also determined. Quantitative features are summarized in Table 2. In these experiments, the injection time was 8 s, LOD and LOQ were determined starting from a 2 mg/ml solution of polymyxin B. When 2 mg/ml solution is injected for 8 s, selectivity is good, except for S_3 which tends to decrease substantially. Compared to LC, the LOQ of polymyxin B_1 obtained in CE was in the same range. This should be due to the benefit of CE using a detection wavelength below 200 nm without influence of background absorption.

In addition, three commercial samples were analyzed and the results are summarized in Table 3.

Table 3
Composition of three commercial samples [a]

Component	Contents (RSD, %) [b]		
	Sample 1	Sample 2	Sample 3
B_1	53.8 (1.5)	61.7 (0.1)	73.7 (1.7)
B_2	25.7 (2.2)	20.3 (1.9)	6.6 (2.2)
B_3	7.4 (2.7)	3.8 (2.1)	2.4 (2.7)
B_4	1.7 (1.2)	1.0 (1.5)	[c]
Other impurities (sum) [d]	11.4 (0.3)	12.9 (3.6)	17.0 (3.0)

[a] Conditions as in Fig. 4, each sample was injected in triplicate and the average value is listed.
[b] Corrected peak area was employed to normalize the contents based on the assumption that all the components have the same response factor.
[c] The content was too low to be detected.
[d] Disregard level was 0.2%.

4. Conclusion

A CZE method was developed for polymyxin B analysis. It was established that M-β-CD and IPA as buffer modifiers were necessary for selectivity improvement. The overall optimal separation conditions were obtained using a central composite experimental design. Under optimal conditions, the separation selectivity is comparable to that of LC, but the

analysis time is about three times shorter than with the latter. It was established that the method is robust and can be used for quantitative analysis of polymyxin B.

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